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PRINCIPAL INVESTIGATOR: Matthew D. Westfall
Jennifer A. Pietenpol, Ph.D.

CONTRACTING ORGANIZATION: Vanderbilt University Medical Center
Nashville, Tennessee 37232-2103

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Matthew D. Westfall
Jennifer A. Pietenpol, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Vanderbilt University Medical Center
Nashville, Tennessee 37232-2103

E-Mail:
Matthew@toxicology.mc.vanderbilt.edu

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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

p63 is a recently identified homolog of p53 that is found in the basal layer of several epithelial tissues such as the epidermis, oral mucosa, prostate, urogenital tract, and **mammary gland**. Studies with p63-/- mice and analysis of several human autosomal dominant disorders with germline p63 mutations suggest p63 involvement in maintaining epithelial stem cell populations. However, the biochemical mechanisms by which p63 functions are not well understood. The objective of the current study is to determine the splice variants that are expressed in primary human mammary epithelial cells (HMECs) and the biochemical activity p63 has in these epithelial cell populations. Progress to date includes (i) cloning of p63 splice variants and development of assay systems in primary epidermal cell cultures to analyze p63 expression and biochemical activity; (ii) determining that p63 represses transcription and binds directly to p53 consensus sites in the p21 and 14-3-3σ promoters *in vitro* and *in vitro*; (iii) development of optimal growth conditions for primary human mammary epithelial cells. It is critical to obtain a more thorough understanding of how p63 works at the molecular level in HMECs to better understand the role of p63 in breast cancer development.

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INTRODUCTION

As stated in my original grant proposal, the overall goal of my project was to increase our knowledge of the checkpoint controls involved in preventing transformation of normal breast epithelial cells. Specifically, I proposed to elucidate the role of the Chk1 kinase in cell cycle checkpoint function. **The following two-part hypothesis was put forth. First, human Chk1 kinase is activated by genotoxic stress and its activity is required for G2 checkpoint function in breast epithelial cells. Second, ablation of Chk1 function in breast cancer cells will abrogate activation of the G2 checkpoint response and sensitize cells to anticancer agents that induce DNA damage.**

An integral aspect of my research proposal was development of a Chk1 kinase assay from breast epithelial cells (specific aim#1). Unfortunately, an optimal assay was never achieved. Consequently, the inability to reliably test Chk1 activity made continuation with projects to ablate Chk1 expression or inhibit Chk1 activity (specific aim#2) ultimately unproductive at the time. After serious consideration, my advisor and Ph.D. Committee suggested I pursue another avenue of research.

Our laboratory has a strong interest in analyzing the regulation and biochemical activity of p53 in mammary epithelial cells, both normal and transformed. p53 is a key transcriptional regulator of genes, the products or which are involved in cell cycle checkpoints. Since disruption of the p53 signaling pathway is the most common genetic alteration in breast cancer, understanding p53-signaling and how it regulates normal epithelial cell function will no doubt continue to be significant (13). Recent studies have shown that a p53 family member, p63, plays an important role in p53 signaling in the breast as described below.

The importance of p63 function in mammalian epithelial cells was first noticed with generation of $p63^{-/-}$ mice. **The $p63^{-/-}$ mice are born, but die shortly after birth, and are completely deficient in mammary gland development (11,27).** Subsequent studies showed p63 is expressed in breast myoepithelial cells and certain subsets of breast neoplasias (2,25). Because p63 has been implicated in the maintenance of the stem cell population in the basal layer of stratified epithelium, we hypothesize that p63 may function as a dominant negative in suppressing p53 signaling in numerous epithelial tissues, including the mammary gland. In support of this, we found several splice variants of p63 RNA expressed in normal keratinocytes and tumor cells of the head and neck. However, only one of these splice variants (ΔNp63α), which lacks a transactivation domain, was found to be expressed at the protein level. Several studies have suggested this dominant-

negative role for p63 regulation of p53 signaling, but conclusive data demonstrating p63 binding to p53 consensus DNA sites *in vivo*, and negative regulation of p53 mediated transcription, has only recently been shown.

I am currently focusing on evaluating the effect of p63 on p53 signaling, as well as identifying novel p63 regulated genes, in normal and tumor derived mammary epithelium. Elucidation of the p63 mechanism of action in this model system will provide a better understanding of the development of epithelial tumors in tissues which express p63 such as breast, cervix, prostate, and skin.

BODY

Revised Background and Hypothesis

Recently p63 and p73, two p53 homologs, were identified (1,10,12,23,26). These proteins exhibit a high sequence and structural homology to the p53 protein. Each gene encodes an amino-transactivating domain, a core DNA-binding domain, and a carboxy-oligomerization domain. However, there are significant differences between these homologs and p53. Both p63 and p73 genes contain two transcriptional start sites that are used to generate transcripts that encode proteins with or without an amino-transactivating domain. Proteins with the transactivating domain are termed TAp63 or TAp73 and proteins lacking the transactivation domain are termed ΔNp63 or ΔNp73. Additionally, both genes are alternatively spliced to generate proteins with different carboxy-termini. For example, six splice variants can be generated from the two promoters of the p63 gene with three different C-termini termed α, β, and γ (4,26). The p63α and p73α proteins also contain an additional region not found in p53 known as a sterile alpha motif or SAM domain. This domain is found in the α form of p63 and p73 (26,26) and is a protein-protein interaction domain implicated in developmental processes (18,22).

In addition to the structural differences within the p53 gene family, differing functional properties were discovered. These differences became apparent after analysis of $p63^{-/-}$ and $p73^{-/-}$ mice. Whereas $p53^{-/-}$ mice are developmentally normal but prone to neoplastic disease (8), the $p63^{-/-}$ and $p73^{-/-}$ mice have severe developmental abnormalities. The $p63^{-/-}$ mice are born, but die shortly after birth, and are deficient in the development of limbs and several epithelial tissues. **Specifically, $p63^{-/-}$ mice are completely deficient in mammary gland development** (11,27). The significance of this finding is evident with the recent discovery of germ-line p63 mutations in the human heterozygous, autosomal dominant disorder limb-mammary syndrome characterized by mammary gland hypoplasia (24).

The p63 protein is localized to the nucleus of basal cells of stratified epithelia

such as skin, oral mucosa, cervix, vaginal epithelium, urothelium, prostate, breast, and other tissues (5,6,26). The ΔNp63α splice variant is the predominant, if not the only, form expressed in these basal epithelial cells (6,14,26). Interestingly, expression of p63 protein in breast epithelium was found in myoepithelial cells which constitute the basal cell layer of normal mammary epithelia (2). This suggests a role for p63 in mammary gland development and maintenance of the proliferative potential of the basal cell layer. Subsequent studies have further examined p63 in the breast and its possible role in neoplastic development. DiRenzo *et al.* found that an immortalized mammary epithelial cell line, generated by ectopic telomerase expression, expressed the ΔNp63α protein (7). Wang *et al.* showed p63 expression in myoepithelial cells of normal breast and in ductal hyperplasia (25). However, they also found that p63 was rarely expressed in carcinoma *in situ* and not expressed in invasive carcinomas. These studies suggest a role for p63 in the initial stages of breast neoplasia. These data are supported by the studies of Parsa *et al.* and Pellegrini *et al.* who showed that a decrease in p63 was associated with a reduced proliferative potential and subsequent terminal differentiation of skin keratinocytes (14,15). Additional biochemical studies showed ectopic expression of the ΔNp63-splice variants can decrease p53 target gene promoter activity suggesting a role for ΔNp63α in maintaining the proliferative capacity of cells by repressing p53 target genes involved in growth arrest (10,26,28). **Thus, we hypothesize that p63 sustains the proliferative capacity of the basal cells in normal human mammary epithelium through sequence-specific DNA binding to promoter regions of select growth regulatory genes.**

Since p63 has been localized to the basal cell layer of most stratified squamous epithelial tissues, we chose to use primary human epidermal keratinocytes (HEKs) as our initial model system. We chose this model system for three reasons. First, we wanted to examine p63 in the context of a primary/normal cell for elucidating its function. Second, due to the similarities between the $p63^{-/-}$ mouse and human syndromes, we feel that understanding p63 function and regulation in our system will be applicable to other primary epithelial systems. Third, epidermal keratinocyte availability and cell culture techniques are far greater than normal human mammary epithelial cells. This was the primary factor for initiating our studies using HEKs since HEKs are available through a Vanderbilt core facility for $100 per cryopreserved vial (this can provide approximately 250 10cm dishes of cells). In contrast, primary human mammary epithelial cells (HMECs) cost approximately $500 per cryopreserved vial (this can provide approximately 30-50 10cm dishes). Because we initiated and optimized our studies in HEKs, we are now able to perform similar experiments in HMECs in a more time- and cost-efficient manner.

REVISED TECHNICAL OBJECTIVES

Specific Aim 1: A) To determine the p63 splice variants that are expressed in primary human epidermal cells and how expression changes during differentiation. **B)** To determine if ΔNp63α represses transcription and binds directly to p53 consensus sites in the p21 and 14-3-3σ promoters *in vitro* and *in vivo*. **C)** To formulate growth conditions for primary HMECs.

The following goals and conditions were accomplished and optimized in HEKs and are ongoing in HMECs. See attached manuscript submitted to "Molecular and Cellular Biology"

1) Clone of p63 splice variants expressed in HEKs and determination of p63 splice variant expressed in HEKs (months 1-12)

2) Analyze changes in p63 transcript and protein during differentiation and comparison of growth regulatory genes and their proteins to p63 during differentiation (months 1-12)

3) Analyze the role of ΔNp63α as a transcriptional repressor (months 1-12)

4) Determine by McKay assays that ΔNp63α can bind, *in vitro*, p53 consensus site oligonucleotides (months 1-12)

5) Determine by chromatin immunoprecipitation assays that ΔNp63α can bind, *in vivo*, p53 consensus sites in the p21 and 14-3-3σ promoters (months 1-12)

6) Formulate growth conditions for primary HMECs (months 1-12)

Specific Aim 2: A) To determine the p63 splice variants that are expressed in human mammary epithelial cells and how expression changes during senescence and after genotoxic stress. **B)** To determine if ΔNp63α binds directly to p53 consensus sites in the p21 and 14-3-3σ promoters *in vivo*.

1) Analyze expression of p63 splice variant expressed in HMECs (months 13-16)

2) Analyze changes in p63 transcript and protein during senescence and after genotoxic stress; comparison of growth regulatory genes and their proteins to p63 will also be determined (months 13-16)

3) Determine by chromatin immunoprecipitation assays if ΔNp63α can bind p53 consensus sites in the p21 and 14-3-3σ promoters *in vivo* (months 13-19)

Specific Aim 3: To examine p63 phosphorylation in HMECs during senescence and after genotoxic stress.

1) Analyze phosphorylation of ΔNp63α in HMECs by one- and two-dimensional gel electrophoresis during senescence and after genotoxic stress (months 16-24)

2) Utilize mass spectrometry to identify phosphorylated residues and identify candidate kinases through consensus phosphorylation sites (months 16-24)

3) Generate bacterially expressed ΔNp63α to test candidate kinases (months 13-16)

4) Determine how phosphorylation affects ΔNp63α function in HMECs (subcellular localization, DNA binding, degradation) (months 18-24)

Rationale: Numerous studies have focused on the importance of phosphorylation for regulation of the p63-related tumor suppressor p53 (reviewed in (19)). Additionally, a recent study by Sanchez-Prieto *et al.* determined that a third p53 family member, p73, was phosphorylated through a p38 MAP kinase pathway (16). Phosphorylation has been shown to be important for protein stability and activity of both p53 and p73 (16,19). We have subsequently determined that ΔNp63α is a phosphoprotein in epithelial cells grown in culture (see attached manuscript). Taken together, these data illustrate the importance of phosphorylation and the necessity of understanding how it regulates ΔNp63α.

Methods: For all experiments listed below, HMECs will be analyzed for p63 protein expression during senescence and after genotoxic stress. As described by Brenner *et al.* senescence of HMECs *in vitro* will be determined both morphologically and by increased expression of the cyclin dependent kinase inhibitor p16 (3). For genotoxic stress experiments, HMECs will be treated with adriamycin, 5-fluorouracil, or ionizing radiation and harvested for indicated analyses.

Analyze ΔNp63α phosphorylation: HMECs will be grown until senescence or treated with the panel of genotoxic agents. Protein extracts from control, senescent, and treated cells will be assayed for phosphorylation status of ΔNp63α by 1-D or 2-D SDS-PAGE and Western analysis as routinely performed in our lab (17,20). As a control/standard for evaluating p63 in 2-D analysis, we will use a truncated p53 protein isolated from yeast (20). **Identify phosphorylated residues:** After optimization of conditions to separate differentially phosphorylated p63, mass spectrometry will be utilized to identify specific phosphorylated residues. To increase sensitivity, protein extracts will be fluorescently labeled and subsequently evaluated in the Vanderbilt University Proteomics core facility. The core facility has resources devoted exclusively to analyzing proteins separated by 2-D SDS PAGE which will greatly aid in identifying p63 phosphorylated residues.

Generate bacterially expressed ΔNp63α: ΔNp63α will be subcloned into the pET-28a (Novagen) bacterial expression vector to generate a 3' 6X histidine tagged protein. The purified protein will be used as a substrate in kinase assays to verify candidate kinases, indicated by consensus phosphorylation sequences, identified by mass spectrometry of ΔNp63α as described above.

Determine effect of phosphorylation: Effect of phosphorylation on ΔNp63α will be examined by three methods: subcellular localization, DNA binding, and protein stability. For all experiments cells will be treated as described above. Subcellular localization of ΔNp63α will be determined by centrifugal fractionation of cell extracts or immunohistochemistry as routinely performed in our lab (9). DNA binding assays will be performed on immunoprecipitated p63 from cell extracts as routinely performed in our lab ((21) and attached manuscript). Protein stability assays well be performed using ^{35}S-methionine in pulse-chase experiments to determine protein half-life.

KEY RESEARCH ACCOMPLISHMENTS

•Cloned and subsequently analyzed p63 expression in a primary epithelial cell model system.

•Determined that ΔNp63α functions as a transcriptional repressor.

•Determined through *in vitro* and *in vivo* DNA binding assays that ΔNp63α bound to both p53 consensus binding sites in the 14-3-3σ and p21 promoters.

•Determined that ΔNp63α is a phospho-protein.

•Optimized growth conditions for human mammary epithelial cells.

REPORTABLE OUTCOMES

•Westfall, M.D., Mays, D.J., Sniezek, J.C., Pietenpol, J.A. (2002) The ΔNp63α Phosphoprotein Binds the p21 and 14-3-3σ Promoters *in vivo* and has Transcriptional Repressor Activity that is Reduced by Hay-Wells Syndrome-Derived Mutations. Molecular and Cellular Biology (submitted for publication)

CONCLUSIONS

The main objective of this research is to test the hypothesis that **p63 sustains the proliferative capacity of the basal cells in normal human mammary epithelium through sequence-specific DNA binding to promoter regions of select growth regulatory genes.** Primarily, our research goal is to determine the biochemical activity of p63 in human mammary epithelial cells and determine if this activity contributes to breast cancer development. The results described above and in the attached manuscript suggest that ΔNp63α functions to repress growth inhibitory genes which are upregulated by the tumor suppressor p53 in response to genotoxic stress. Evidence exists that breast tumor cells with defective DNA damage checkpoint function (usually due to mutation or loss of the p53 gene) have increased sensitivity to anticancer agents. Thus it is critical to understand the molecular basis for ΔNp63α function and regulation since ΔNp63α may function to

repress growth inhibitory genes in breast cancer cells. p63 activity could potentially dictate the type of therapy breast cancer patients recieve. Additionally, the results from our studies will need to be evaluated in the context of p53 status. Since p53 is one of the most frequent genetic alterations found in breast cancer, it will be important to determine if p63 activity is involved in the development of breast cancer in cells with or without wild-type p53.

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APPENDIX

Content: 1 submitted manuscript

Westfall, M.D., Mays, D.J., Sniezek, J.C., Pietenpol, J.A. (2002) The ΔNp63α Phosphoprotein Binds the p21 and 14-3-3σ Promoters *in vivo* and has Transcriptional Repressor Activity that is reduced by Hay-Wells Syndrome-Derived Mutations. Molecular and Cellular Biology (submitted for publication)

The ΔNp63α Phosphoprotein Binds the p21 and 14-3-3σ Promoters *In Vivo* and has Transcriptional Repressor Activity that is Reduced by Hay-Wells Syndrome-Derived Mutations

Matthew D. Westfall[1], Deborah J. Mays[1], Joseph C. Sniezek[1,2], and Jennifer A. Pietenpol[1,*]

[1]Department of Biochemistry, Center in Molecular Toxicology, The Vanderbilt-Ingram Comprehensive Cancer Center, Vanderbilt University School of Medicine, Nashville, Tennessee 37232, USA.

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*Corresponding Author: Mailing address: Department of Biochemistry, The Vanderbilt-Ingram Comprehensive Cancer Center, Vanderbilt University School of Medicine, Nashville, TN 37232. Phone (615) 936-1512. Fax: (615) 936-2294. E-mail: jennifer.pietenpol@vanderbilt.edu

[2]Current Address: Head and Neck Oncologic Surgery, MCHK-DSH, 1 Jarrett White Road, Tripler Army Medical Center, Honolulu, HI 96859

Abstract

p63 is a recently identified homolog of p53 that is found in the basal layer of several stratified epithelial tissues such as the epidermis, oral mucosa, prostate, and urogenital tract. Studies with $p63^{-/-}$ mice and analysis of several human autosomal dominant disorders with germline p63 mutations suggest p63 involvement in maintaining epidermal stem cell populations. The p63 gene encodes six splice variants with reported transactivating or dominant-negative activities. The goals of the current study were to determine the splice variants that are expressed in primary human epidermal keratinocytes (HEKs) and the biochemical activity p63 has in these epithelial cell populations. We found that the predominant splice variant expressed in HEKs was ΔNp63α and it was present as a phosphorylated protein. During HEK differentiation, ΔNp63α and p53 levels decreased while expression of p53 target genes p21 and 14-3-3σ increased. ΔNp63α had transcriptional repressor activity *in vitro* and this activity was reduced in ΔNp63α proteins containing point mutations, corresponding to those found in patients with Hay-Wells syndrome. Further, we showed that ΔNp63α and p53 can bind the p21 and 14-3-3σ promoters *in vitro* and *in vivo,* with decreased binding of p63 to these promoters during HEK differentiation.

These data suggest that ΔNp63α acts as a transcriptional repressor at select growth regulatory gene promoters in HEKs and this repression likely plays an important role in the proliferative capacity of basal keratinocytes.

Introduction

Recently p63 and p73, two p53 homologues, were identified (2,25,35,47,50). These proteins exhibit a high sequence and structural homology to the p53 protein. Each gene encodes an amino-transactivating domain, a core DNA-binding domain, and a carboxy-oligomerization domain. However, there are significant differences between these homologues and p53. Both p63 and p73 genes contain two transcriptional start sites that are used to generate transcripts that encode proteins with or without an amino-transactivating domain. Proteins with the transactivating domain are termed TAp63 or TAp73 and proteins lacking the transactivation domain are termed ΔNp63 or ΔNp73. Additionally, both genes can be alternatively spliced to generate proteins with different carboxy-termini. For example, six splice variants can be generated from the two promoters of the p63 gene with three different C-termini termed α, β, and γ (8,50). The p63α and p73α proteins also contain an additional region not found in p53 known as a sterile alpha motif or SAM domain. This domain is found in the α form of p63 and p73 (25,50) and is a protein-protein interaction domain implicated in developmental processes (39,46).

In addition to the structural differences within the p53 gene family, differing functional properties were discovered. These differences became

apparent after analysis of $p63^{-/-}$ and $p73^{-/-}$ mice. Whereas $p53^{-/-}$ mice are developmentally normal but prone to neoplastic disease (14), the $p63^{-/-}$ and $p73^{-/-}$ mice have severe developmental abnormalities. The $p63^{-/-}$ mice are born, but die shortly after birth, and are deficient in the development of limbs and several epithelial tissues such as skin, prostate, mammary gland, and urothelia (32,51). The $p73^{-/-}$ mice exhibit neurological, pheromonal, and inflammatory defects (52).

The p63 protein is localized to the nucleus of basal cells of stratified epithelia such as skin, oral mucosa, cervix, vaginal epithelium, urothelium, prostate, breast, and other tissues (12,13,50). The ΔNp63α splice variant is the predominant, if not the only, form expressed in these basal epithelial cells (13,36,50). Ectopic expression of the ΔN-splice variants can decrease p53 target gene promoter activity suggesting a role for ΔNp63α in maintaining the proliferative capacity of cells by repressing p53 target genes involved in growth arrest (25,50,54). This hypothesis is supported by the data of Parsa *et al.* and Pellegrini *et al.* showing that a decrease in p63 was associated with a reduced proliferative potential and subsequent terminal differentiation of skin keratinocytes (36,37).

Herein, we analyzed the role of p63 in primary human epidermal keratinocyte (HEK) differentiation. Our results indicate that ΔNp63α is the

predominant form of p63 protein expressed in primary cultures of HEKs and is down-regulated during differentiation. We also show that ΔNp63α is a phosphoprotein that can function as a transcriptional repressor and bind consensus p53-binding sites in the $p21^{waf1}$ (p21) and 14-3-3σ promoters *in vivo*.

Materials and Methods

Cell Culture and Treatment. Second passage primary human epidermal keratinocytes (HEK) were obtained from the Vanderbilt Skin Disease Research Core. HEKs were isolated as previously described (17) and were cultured in EpiLife M-EPI-500 keratinocyte growth media (Cascade Biologics, Portland, OR) supplemented with human keratinocyte growth supplement S-001-5 (Cascade Biologics) and 0.06 mM $CaCl_2$. The human colorectal carcinoma cell lines HCT116 and RKO were cultured in DMEM medium supplemented with 10% FCS and 1% penicillin-streptomycin. The human embryonic kidney cell line 293 was kindly provided by S. Hiebert (Vanderbilt University Department of Biochemistry, Nashville, TN) and cultured in DMEM medium supplemented with 10% FCS and 1% penicillin-streptomycin. All cells were cultured at 37°C with 5% CO_2.

p63 Cloning. ΔNp63 splice variants, α, β, and γ were cloned from primary human oral mucosa. Tissue was obtained from patient specimens collected at Vanderbilt University School of Medicine and the Access RT-PCR System (Promega, Madison, WI) was used with mRNA isolated from the primary human oral keratinocytes. Primers used to generate the splice variants were as follows: ΔNp63 N-terminal 5'-CCCAAGCTTAATAC GACTCACTATAGGGAGACCATGGAACAAAAACTCATCTCAGAAGAGGATCTGATG TTGTACCTGGAAAACAATG–3'; ΔNp63α C-terminal 5'–CGGGATCC TCACTCCC CCTCCTCTTTG–3'; ΔNp63β C-terminal 5'–CGGGATCCTCAGACTTGCCAGATCC TG–3'; ΔNp63γ C-terminal 5'–CGGGATCCCTATGGGTACACTGATCGG–3'. p63 splice variants were subsequently cloned into the pCEP4 vector (Invitrogen, Carlsbad, CA) for transient transfections.

HEK Differentiation Assays. HEKs were grown to ~90% confluency, washed twice with PBS, and induced to differentiate by the addition of 1 mM $CaCl_2$ in growth factor-deficient medium. Cells were harvested at days 0, 2, 4, 6, and 8 after the induction of differentiation. Culture medium was changed every two days. Cells were harvested as described for Western and Northern analyses.

Protein Lysate Preparation and Western analysis. 100 mm dishes of primary HEKs were washed twice with ice-cold phosphate-buffered saline, and harvested by scraping into 750 μl Kinase Lysis Buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Nonidet P-40, 0.1% Triton X-100, 4 mM EDTA, 1 mM DTT) containing the phosphatase inhibitors 50 mM NaF, 0.2 mM Na-vanadate, 10 mM p-Nitrophenyl phosphate, and 10 mM β-Glycero-Phosphate and the protease inhibitors antipain (10 μg/ml), leupeptin (10 μg/ml), pepstatin A (10 μg/ml), chymostatin (10 μg/ml) (Sigma), and 4-(2-aminoethyl)-benzenesulfonylfluoride (200 μg/ml) (Calbiochem, San Diego, CA). Cells were incubated on ice 30-45 min, and the protein supernatant was clarified by centrifugation at 13,000 x *g* for 10 min at 4°C. Protein concentration was determined by the Bio-Rad Protein Quantification kit (Bio-Rad Laboratories, Hercules, CA). Western analysis was performed as previously described (17) using the following primary antibodies: α-p63 monoclonal antibody Ab-1 (Oncogene Research Products, Calbiochem) α-p53 monoclonal antibody 1801 (Oncogene Research Products, Calbiochem), α-$p21^{Waf1}$ antibody Ab-1 (Oncogene Research Products, Calbiochem), α-14-3-3σ polyclonal antibody N-14 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), α-β-actin polyclonal antibody I-19 (Santa Cruz Biotechnology Inc.). Uniformity of protein loading was assessed by β-actin

analyses as well as fast green staining of the membranes.

Northern analysis. 150 mm dishes (100 mm for adenovirus transduction) of HEK cells were harvested for mRNA isolation as previously described (18). mRNA (1-3 μg) was lyophilized, resuspended in sample buffer [1x morpholine-propanesulfonic acid (MOPS; 0.1 M MOPS pH 7.0, 40 mM sodium acetate, 5 mM EDTA pH 8.0), 50% formamide, 6.5% formaldehyde] and heated at 55°C for 15 min. A 10X loading buffer (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol, 0.3 mg of ethidium bromide per ml) was added to the sample at a 1X concentration, and mRNA was resolved by gel electrophoresis on a 1% agarose gel containing 2% formaldehyde and 1X MOPS. The gel was washed 2 x 40 min in 10x SSC buffer (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer, and mRNA was transferred to a supported nitrocellulose membrane (Gibco BRL). MDM2, ΔNp63, p53, 14-3-3σ, p21 and cyclophilin cDNAs were labeled with [α-^{32}P] dCTP using Prime-It II (Stratagene, La Jolla, CA). After a 2 h prehybridization in Express Hyb (Clontech Laboratories, Inc., Palo Alto, Calif.), membranes were incubated 1 h with 1 x 10^6 cpm of labeled cDNA per ml in Express Hyb. Membranes were washed 2 x 1 min at room temperature in 0.1x SSC/0.1% SDS, followed by 1 hour in 0.3x SSC/0.1% SDS. Blots were subsequently exposed for autoradiography.

Potato Acid Phosphatase Assay. HEK protein lysates (75 μg) were incubated in 50 mM PIPES, pH 6.0, with 1 mM DTT (final volume 100 μl) for 10 min at 30 °C, followed by addition of 0.5 unit of potato acid phosphatase (Sigma). The incubation was continued for 30 min at 30 °C. The phosphatase reaction was stopped by the addition of Laemmli SDS sample buffer. The control and phosphatase-treated lysates were analyzed by Western.

Luciferase Assays. HCT116 cells were transiently transfected with the p21-luciferase reporter construct (kindly provided by B. Vogelstein, Johns Hopkins Oncology Center, Baltimore, MD) (15), and p53 or Gal4-p63 fusion proteins. 293 cells were transiently transfected with a Gal4-TK-luciferase reporter construct (kindly provided by S. Hiebert) previously described (40), and Gal4-p63 fusion constructs. Gal4-p63 fusion cDNAs were cloned into the pM2/pY2 vector at the *SalI-HindIII* sites. The following primers were used to generate the Gal4-p63 cDNAs: Gal4-ΔNp63 N-terminal 5'-ACGCGTCGACTTGTACCTGGAAAACAATG-3'; ΔNp63α C-terminal 5'-CCC AAGCTTTCACTCCCCCTCCTCTTTG–3'; ΔNp63β C-terminal 5'-CCCAAGCTTTCAGACTTGCCAGATCCTG-3'; ΔNp63γ C-terminal 5'-CCCAAGCTTCTATGGGTACACTGATCGG– 3'; Gal4- SAM N-terminal

5'-ACGCGTCGACCCTCCGTATCCCACAGAT-3'; Gal4-SAM C-terminal 5'-CCCAAGCTTTCAGAATTCGTGGAGCTGCCG-3'. Gal4-ΔNp63α SAM-domain point mutants (31) (kindly provided by H. van Bokhoven, Department of Human Genetics, University Hospital Nijmegen, The Netherlands) served as templates to generate additional Gal4-fusion cDNAs using the above primers. All transfections were performed using Lipofectamine (Gibco BRL) and cells were harvested 48 h after transfection. Luciferase activity measurements were performed using the Dual-Luciferase Assay Kit (Promega).

DNA Binding Assay. Radiolabeled oligonucleotide duplexes containing the two p53 DNA binding sites of p21 and 14-3-3σ were generated using the following oligonucleotides: p21 site 1, 5'-TGGCCATC AGGAACATGTCCCAACATGTTGAGCTCTGGCA -3'; p21 site 2, 5'-TAGAGGAAG AAGACTGGGCATGTCTGGGCAGAGATTTCCA-3'; 14-3-3σ site 1, 5'- CTGGGA CTACAGGCATGTGCCACCATGCCCGGCTAATTTT-3'; 14-3-3σ site 2, 5'-TGGA AACCCTGTAGCATTAGCCCAGACATGTCCCTACTCCTCC-3'. For end-labeling, 500 ng of each oligonucleotide were incubated with 167 μCi of [γ-^{32}P]-ATP and T4 Polynucleotide Kinase (New England Biolabs, Beverly, MA) in Kinase Buffer (70 mM Tris pH 7.6, 10 mM $MgCl_2$, 5 mM DTT) for 2 h at 37°C. DNA was ethanol precipitated two times, allowed to air dry, and resuspended in

100 μl of Annealing Buffer (20 mM Tris pH 7.5, 2 mM $MgCl_2$, 50 mM NaCl). The complementary oligonucleotides for each binding site were mixed, boiled for 5 min, and allowed to anneal.

The human large cell lung carcinoma cell line H1299, which does not express p53 or p63 protein, was transfected with pCEP4 expression vectors encoding either myc-tagged p53 or myc-tagged p63 proteins or with the empty pCEP4 expression vector (Invitrogen). Western analysis of the transfected cell lysates using α-myc antibody was performed and p53 and p63 quantified in triplicate using a Fluor-S MAX MultiImager (Bio-Rad Laboratories, Hercules, CA). Equivalent amounts of p53 and p63 protein were immunoprecipitated by rocking at 4°C for 1 h in DNA Binding Buffer (DBB: 20 mM Tris pH 7.2, 100 mM NaCl, 10% glycerol, 1% Nonidet P-40) using the myc antibody and 15 μl bed volume of protein A sepharose (PAS) (Amersham Biosciences Corp., Piscataway, NJ). Immunoprecipitated proteins were washed twice with DBB, and once in DBB containing 1 mM DTT. The immunopurified protein was rocked for 1 h at 4°C in 100 μl of DBB with 2 x 10^6 cpm of [α-^{32}P]-labeled DNA fragments prepared as described above. The protein-DNA complexes were rocked for 1 h at 4°C with 10 μg poly[dIdC] (Roche Molecular Biochemicals, Indianapolis, IN.). After three washes with DBB, proteins were digested with SDS/Proteinase K (VWR Scientific

Products, West Chester, PA) in TE8 (20 mM Tris pH 8.0, 10 mM EDTA) for 30 min at 55°C prior to electrophoresis on 10% polyacrylamide gels (acrylamide:bis acrylamide, 19:1) at 40 V. Radiolabeled DNA was quantified using an Instant Imager (Packard Instrument Company, Downers Grove, IL).

Formaldehyde cross-linking. Growth medium was aspirated from ~5 x 10^6 cells and cell cultures were washed with phosphate-buffered saline (PBS) and incubated with a 1.6% formaldehyde (EM Science) solution in PBS for 13 min at room temperature. The cross-linking was terminated by the addition of glycine to a final concentration of 0.144 M for 5 min. Monolayers were washed twice with PBS. Extracts were prepared by scraping cells in 1 ml of RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, 5 mM EDTA) containing the protease inhibitors antipain (10 μg/ml), leupeptin (10 μg/ml), pepstatin A (10 μg/ml), chymostatin (10 μg/ml), 4-(2-aminoethyl) benzenesulfonylfluoride (200 μg/ml), and the phosphatase inhibitors 50 mM NaF and 0.2 mM Na-vanadate. Cell lysates were sonicated to yield chromatin fragments of approximately 600 bp as assessed by agarose gel electrophoresis. Debris was pelleted by centrifugation for 15 min at 13,000 x *g*. The lysate was aliquoted and 0.8 mg of protein extract were precleared with 10 μg of mouse immunoglobulin G bound to PAS for p53 immunoprecipitation or 20 μg of rabbit immunoglobulin

G bound to PAS for p63 immunoprecipitation. Protein lysates were precleared for 1 hr at 4°C. After centrifugation for 2 min at 13,000 x *g*, supernatants were transferred to a new tube. A 15 μl bed volume of PAS and 2 μg of α-p53 antibody (1801 Oncogene Research Products) or α-p63 antibody (H129 Santa Cruz Biotechnology) were added to extracts precleared with non-specific antibodies, and immunoprecipitation was performed by rocking overnight at 4°C. To control for nonspecific binding during immunoprecipitation, cross-linked lysates were also immunoprecipitated with mouse monoclonal α-cyclin B1 (GNS1 Santa Cruz) or rabbit polyclonal α-Bax (N20 Santa Cruz) antibodies that did not cross-react with p53 or p63 respectively.

Immunocomplexes were washed twice with RIPA buffer, four times with IP wash buffer (100 mM Tris pH 8.5, 500 mM LiCl, 1% Nonidet P-40, 1% deoxycholic acid), and twice more with RIPA buffer. Between washes, samples were rocked for 5 min at 4°C; 200 μl of cross-linking reversal buffer (125 mM Tris pH 6.8, 10% β-mercaptoethanol, 4% SDS) were added to the washed PAS pellet. Samples were boiled for 30 min to reverse the formaldehyde cross-links. DNA was phenol-chloroform extracted, the phenol-chloroform phase back extracted with 10mM Tris pH 8.3, ethanol precipitated, allowed to air dry, and dissolved in sterile H_2O.

PCR amplification. p21 Site 1 and 14-3-3σ Site 1 PCR amplifications were performed in 16.6 mM $(NH_4)_2SO_4$, 0.67 mM Tris pH 8.8, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 10% dimethyl sulfoxide, 1.5 mM nucleotides, and 1.25 U Taq polymerase (Promega). Per 25 μl reaction, 175 ng of each primer were used. Forty-five PCR cycles were performed for p21 site 1, each cycle consisting of 20 sec at 94°C, 45 sec at 61°C, and 25 sec at 72°C. 14-3-3σ site 1 was amplified using 45 PCR cycles each consisting of 20 sec at 94°C, 45 sec at 57.5°C, and 25 sec at 72°C. p21 Site 2 and 14-3-3σ site 2 PCR amplifications were performed using Ready-To-Go PCR beads (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's directions with a final primer concentration of 0.4 μM. Thirty PCR cycles were performed for p21 site 2. Each cycle consisted of 30 sec at 95°C, 45 sec at 66°C, and 25 sec at 72°C. Thirty-eight PCR cycles were performed for 14-3-3σ site 2. Each cycle consisted of 20 sec at 94°C, 45 sec at 61°C, and 25 sec at 72°C. GAPDH PCR amplification was performed in 10mM Tris pH 9.0, 50mM KCl, 0.1% Triton-X, 0.5 mM $MgCl_2$, 0.25 mM nucleotides, and 1.25 U Taq polymerase (Promega). Each primer was used at 0.2 μM per 25 μl reaction. Thirty-five cycles of PCR were performed for GAPDH amplification, each cycle consisted of 20 sec at 94°C, 45 sec at 62°C and 25 sec at 72°C. Primers used for PCR amplifications

were as follows: for p21 site 1: 5'-GCTTGGGCAGCAGGCTG-3' and 5'-AGCCCTGTCGCAAGGATCC-3'; p21 site 2: 5'-GCAGTGGGGCTTAGAGTGGGG-3' and 5'-CAGGCTTGGAGCAGCTACAATTA C-3'; 14-3-3σ site 1: 5'-CATTTAGGCAGTCTGATTCC-3' and 5'-GCTCACGCC TGTCATCTC-3'; 14-3 3σ site 2: 5'-CTCACTACCTCAAGATACCC-3' and 5'-C ACAGGCCTGTGTCTCCC-3'. GAPDH was amplified using 5'-CACCAGCCATCC TGTCCTCC-3' and 5'-GTTCCTTCCCAGCCCCCACT-3' primers. PCR DNA products were resolved using 8% polyacrylamide gels (acrylamide:bis acrylamide, 19:1) in 1X Tris acetate-EDTA buffer. Gels were stained with ethidium bromide. Relative levels of DNA were determined using Quantity One® software (Bio-Rad Laboratories, Hercules, CA).

Results

ΔNp63α is the predominant splice variant expressed at the protein level in primary human epidermal keratinocytes. To examine the role p63 plays in rapidly growing and differentiating epithelial cells, we analyzed the specific splice variant(s) expressed in the primary cultures of HEKs. Using well characterized conditions for *in vitro* keratinocyte growth (28) we generated protein lysates from HEKs that were rapidly growing or induced to differentiate by removal of growth factors and addition of 1 mM $CaCl_2$. This differentiation resulted in increased protein levels of

keratinocyte differentiation markers loricrin and involucrin (19) as well as the appearance of a differentiated morphology when compared to proliferating cells (Fig. 1A and 1B). In contrast to the increase in differentiation markers, p63 protein levels decreased during differentiation (Fig. 1C). Since p63 expression has been shown to decrease during keratinocyte terminal differentiation *in vivo* (50), our *in vitro* culture system replicated this process.

Previous studies have shown p63 expression in the basal layer of numerous epithelial tissues (50) with the predominant p63 splice variant expressed at the transcript level being ΔNp63α (12,13,34,36,37). Using RT-PCR we generated cDNAs representative of all ΔNp63 splice variants from primary human oral keratinocytes (data not shown). However, when we performed Western analysis to determine p63 levels in protein extracts harvested from rapidly growing and differentiating epidermal keratinocytes, the ΔNp63α splice variant was the predominant form expressed (Fig. 1C).

Expression vectors encoding ΔNp63α, β, γ, and $\Delta Np40^{AIS}$ were generated and transfected into the colon epithelial cell line, RKO, that does not express p63. Protein lysates were generated from transfected RKO cells to serve as controls for the Western analysis. The predominant p63 protein expressed in the HEKs aligned with ectopically expressed ΔNp63α from RKO cells and decreased during differentiation (Fig. 1C). The faster

migrating cross-reactive band on the immunoblot in Fig. 1C, designated with an asterisk, may represent low level ΔNp63β expression. We also examined the p63 transcript levels by Northern (Fig. 1D). We only detected one 4.5kb transcript in mRNA isolated in HEKs, and consistent with our Western results, the level of transcript decreased during differentiation. Due to small differences in cDNA lengths of p63 splice variants, it is possible that the p63 transcript in Fig. 1D represents several splice variants not distinguishable by Northern analysis. The ability to amplify the three splice variants by RT-PCR from primary human keratinocytes supports this conclusion. These results show that ΔNp63α is the predominant p63 splice variant expressed at the protein level in primary cultures of HEKs, either rapidly growing or induced to differentiate.

Western analysis revealed multiple, slower migrating bands that cross-reacted with the p63-specific antibody, suggesting that p63 may also be a phosphoprotein (Figs. 1C and 2A). To determine if the differential migration of ΔNp63α was due to phosphorylation, HEK protein lysates were prepared from differentiated cells (day 4) and treated with potato acid phosphatase in the presence and absence of phosphatase inhibitors (Fig. 2B). Western analysis revealed a collapse of the ΔNp63α protein from several bands to a single band after phosphatase treatment consistent with the conclusion that p63 is a phosphorylated protein.

Changes in expression of cell cycle regulatory proteins and corresponding transcripts in differentiating keratinocytes. As epidermal cells migrate from the basal layer, they begin the process of terminal differentiation characterized by loss of DNA replication and cell cycle arrest, loss of colony forming ability, and an increase in cell size (4,5,44). Upregulation of the p53 target genes p21 and 14-3-3σ are associated with this phenomenon (11,30,33,41,53). p21 is a cyclin-dependent kinase inhibitor originally identified as a p53 target gene and a cyclin/cyclin dependent kinase associated protein (16,21,22,49). 14-3-3σ has been linked to p53-dependent regulation of cell cycle progression at the G2/M transition (7,23).

We evaluated p21 and 14-3-3σ protein and mRNA levels relative to ΔNp63α and p53 levels during HEK differentiation. Both ΔNp63α and p53 proteins decreased 5- and 2-fold, respectively (Fig. 3A, compare RG and day 8). In contrast, 14-3-3σ and p21 protein levels increased by 2- and 2.8-fold, respectively (Fig. 3A). Similar to the changes in protein levels, ΔNp63α and p53 transcript levels decreased during differentiation (Fig. 3B, compare RG and day 8) and p21 and 14-3-3σ increased as cells differentiated (Fig. 3B). These data suggest that increases in 14-3-3σ and p21 expression during keratinocyte differentiation are due to either increased activity of the remaining p53 protein or loss of ΔNp63α-mediated transcriptional repression

resulting from decreased ΔNp63α protein levels.

ΔNp63α represses transcription. Previous studies have shown that ectopic expression of p63 can repress transcription from reporter vectors containing either artificial p53 consensus DNA binding sites or promoter regions from known p53 target genes (8,50). However, the mechanism by which this occurs has not been determined. To explore this mechanism, we analyzed the ability of ΔNp63 splice variants to repress transcription independent of DNA binding. We generated expression vectors that encode Gal4-ΔNp63α, β, and γ fusion proteins (Fig. 4A). To verify the Gal4-fusion did not impair ΔNp63 activity, we tested the ability of the Gal4-ΔNp63α to repress transcription from a p21-luciferase vector (Fig. 4B). We transiently transfected HCT116 cells with expression vectors encoding ΔNp63α or Gal4-ΔNp63α, as well as the p21-luciferase vector, and found that Gal4-ΔNp63α functions similarly to wild type ΔNp63α in its ability to inhibit transcription from the p21 promoter (Fig. 4B). Transfection of p53 served as a positive control for activation of the p21 promoter in this assay.

After establishing that the Gal4 DNA binding domain did not impair ΔNp63α function, we analyzed the Gal4-ΔNp63α, β, and γ splice variants for their ability to repress transcription independent of DNA binding. Each

Gal4-fusion expression vector was transfected into 293 cells and the encoded protein analyzed for its ability to repress luciferase reporter gene transcription regulated by four Gal4 binding sites upstream of a thymidine kinase promoter (Fig. 4A). The ΔNp63α splice variant repressed the TK-luciferase reporter construct ~20-fold whereas the ΔNp63β did not repress transcription and the ΔNp63γ repressed transcription only ~5-fold (Fig. 4C). Transfection of Gal4-ETO2, a previously described transcriptional co-repressor protein (1), served as a positive control for repression of the luciferase reporter vector. These data demonstrate that ΔNp63α can repress transcription directly or through recruitment of transcriptional corepressor molecules.

Analysis of SAM domain mutations. Recent genetic studies link p63 to proper development of limbs, ectodermal appendages, lip and palate in humans (6,31). Specifically, the autosomal dominant disorder, ankyloblepharon-ectodermal dysplasia-clefting (AEC) syndrome or Hay-Wells syndrome, is characterized by point mutations within the SAM domain of p63α splice variants (31). To further explore a potential role of the SAM domain in transcriptional repression, we generated expression vectors encoding Gal4-ΔNp63α fusion proteins containing SAM domain point mutations corresponding to those mutations found in the p63 gene of

individuals with Hay-Wells syndrome. The four point mutations, L459F, G475V, T478P, Q481L, and a corresponding wild-type fusion expression vector were generated using murine p63 (Fig. 4A). The murine p63 was previously used for analysis of SAM domain point mutations by McGrath *et al.* (31). As before, each vector was transiently transfected into 293 cells and analyzed for its ability to repress transcription from the Gal4 DNA binding site reporter vector shown in Fig. 4A. Wild-type murine ΔNp63α repressed transcription ~13-fold (Fig. 4D). This level of repression activity is similar to that observed with human ΔNp63α protein (Fig. 4C). In the context of full-length ΔNp63α, the SAM domain point mutants repressed transcription ~7- to 10-fold suggesting these mutations result in a reduction of p63 activity rather than complete loss of function. Because of the reduced activity of the SAM domain mutant proteins, we generated and analyzed a human Gal4-SAM fusion protein for analysis in the repression assay. Results in Fig. 4 indicate that the SAM domain alone is not sufficient to repress transcription but is likely required for repressor activity in the context of full-length ΔNp63α.

Relative binding affinity of ΔNp63α and p53 to p53 consensus DNA binding sites. The p53 protein binds DNA in a sequence-specific manner and regulates transcription of gene products

involved in processes such as growth arrest, DNA repair, and apoptosis (42). To compare the relative affinity of ΔNp63α and p53 binding to known p53 binding sites, *in vitro* DNA binding assays were performed with radiolabeled duplex oligonucleotides representing p53 binding sites in the p21 and 14-3-3σ promoters as previously described (16,23) (Fig. 5A). H1299 cells were transfected with myc-tagged p53 or myc-tagged ΔNp63α and equal amounts of p53 or ΔNp63α were immunoprecipitated with α-myc epitope antibody. The immunoprecipitates were assayed for their ability to bind the radiolabeled oligonucleotide duplexes. p53 and ΔNp63α bound to both p53 consensus sites present in the p21 promoter (Fig. 5B). Similarly, ΔNp63α bound to both sites present in the 14-3-3σ promoter whereas p53 only displayed significant binding to site 2. Quantification of bound radiolabeled DNA illustrated that p53 has a ~1.5- and ~2-fold greater relative binding affinity than ΔNp63α for the p21 site 1 and site 2, respectively (Fig. 5C). Similarly, p53 had a ~4-fold higher relative binding affinity for the 14-3-3σ binding site 2. In contrast, ΔNp63α bound to the 14-3-3σ site 1 oligonucleotide with a relative binding affinity ~4- to 5-fold greater than p53. Similar assays were performed with the myc-epitope tagged SAM mutants analyzed in Fig. 4. However, comparison of SAM mutants to wild-type ΔNp63α showed no significant difference in relative DNA binding affinity

(data not shown). Thus, the reduction in transcriptional repression activity of the SAM mutations observed in Fig. 4D was not due to differences in DNA binding affinity.

p53 and/or p63 occupancy at the p21 and 14-3-3σ and promoters in vivo during HEK differentiation. Since ΔNp63α exhibited significant binding to p53 consensus sites in the p21 and 14-3-3σ promoters, we studied the ability of p63 to bind these consensus sites *in vivo* using a chromatin immunoprecipitation (ChIP) methodology previously described by our laboratory (45). Either rapidly growing or differentiated cultures (day 8) of HEKs were cross-linked by exposure to 1% formaldehyde as described in Materials and Methods. After cross-linking, p53 and p63 were immunoprecipitated and the DNA to which the proteins bound was purified. The DNA was PCR amplified using primers specific for sequences that flank the p53 response elements in the promoters studied. To assure that the amplified DNA was the correct size, control PCR reactions were performed with or without genomic HEK DNA (Fig. 6A, C, E, and G "+" or "-" lanes respectively). To control for nonspecific binding during immunoprecipitation, cross-linked lysates were immunoprecipitated with mouse monoclonal α-cyclin B1 or rabbit polyclonal α-Bax antibodies that did not cross-react with p53 or p63, respectively (Figs. 6A, C, E, and G; lane C).

The ChIP experiments revealed that p53 and p63 bind both p21 promoter site 1 and site 2 in rapidly growing (RG) and differentiating (day 8) HEKs (Fig. 6A and 6C). p53 occupancy at p21 site 1 increased modestly from rapidly growing cells to differentiation day 8 (less than 1.5-fold) whereas p63 occupancy did not change (Fig. 6B). In contrast to site 1 binding, p53 binding to p21 site 2 did not change and p63 decreased 2-fold (Fig. 6D). Analysis of the 14-3-3σ promoter showed that p53 binding to site 2 remained unchanged in rapidly growing and differentiated keratinocytes (Fig. 6G and 6H); however, there was no appreciable binding of p53 to 14-3-3σ site 1 (Fig. 6E and 6F), consistent with the *in vitro* DNA binding results shown in Fig. 5. Similar to the p21 promoter, p63 bound to both 14-3-3σ site 1 and site 2 *in vivo* and by day 8 of differentiation this binding decreased 2-fold at both site 1 and site 2 (Fig. 6F and 6H). These data suggest that increased p21 and 14-3-3σ expression are due to loss of p63 binding and subsequent decreased transcriptional repression of these promoters by p63.

Discussion

Since the identification of the p53 homologue p63, several studies have investigated its function in epithelial cell growth and development. Using HEKs as a model system, the goal of the current study was to further analyze the biochemical role p63 plays in keratinocytes growth and

differentiation. By using Western and Northern analyses, we demonstrated that the primary splice variant of p63 expressed in HEKs is ΔNp63α, and its expression decreases as cells differentiate. Further, the ΔNp63α protein was present in differentiating HEKs as several phosphoforms. In addition to the reduction in p63 transcript and protein levels during differentiation, we also observed a decrease in p53 transcript and protein. The reduction in p53 and p63 expression correlated with an increase in expression of the cell-cycle regulatory proteins p21 and 14-3-3σ. Using Gal4-fusion proteins, we determined that the p63 protein represses transcription and the ΔNp63α splice variant has the highest activity. In addition, *in vitro* and *in vivo* DNA-binding assays showed that ΔNp63α binds to both p53 response elements in the p21 and 14-3-3σ promoters with p63 occupancy at p21 site 2 and 14-3-3σ site 1 and site 2 decreasing as cells differentiated.

Consistent with previously published reports (36,37), we observed a decrease in p63 transcript and protein during differentiation. Analysis of the limited number of keratinocytes in the $p63^{-/-}$ mouse showed expression of epithelial terminal differentiation markers (51) indicating epithelial defects were due to lack of cell survival and/or proliferation and not impaired terminal differentiation. In support of this, a recent study in zebrafish using RNAi demonstrated that the ΔNp63 splice variant(s) were

required for epithelial proliferation (27). These model systems suggest a role for p63 in maintaining the survival or proliferation of basal keratinocytes, and in conjunction with our HEK data, indicate that loss of ΔNp63α facilitates the growth arrest associated with differentiation.

We determined that p63 migrated as multiple phospho-forms by SDS-PAGE, suggesting that phosphorylation is a mechanism by which p63 protein levels are regulated. This hypothesis is supported by the findings that phosphorylation is a key post-translational modification for regulation of p53 (42). However, ΔNp63α lacks a transactivation domain where many of the regulatory phosphorylation sites are found in p53. Future studies are required to identify the phosphoresidues in ΔNp63α, upstream kinases, and phosphorylation-dependent associated proteins. It has been suggested that ΔNp63α mediated repression can occur through direct protein-protein interaction and several groups have examined the association of p63 splice variants with p53. One possible role of p63 phosphorylation may be in the regulation of p63-p53 binding. Davison *et al.* determined that p63 and p73 can form homo- or hetero-dimers through their oligomerization domain but do not interact with the p53 oligomerization domain (10). Kojima *et al.* found similar results using a yeast two-hybrid system (26). In contrast, several groups have shown that p63 and p53 could interact through the core/DNA-binding domain (20,38,43). Of note, many of these interactions

occur with mutant p53 proteins and the study by Ratovitski *et al.* showed p53-dependent caspase cleavage of ΔNp63α (38). The significance of these findings and the role of protein phosphorylation remains to be determined in the context of proliferating and differentiating epithelial cells.

Since ΔNp63α has been identified as the primary splice variant expressed in epithelial cells, several questions remain to be addressed. In particular, what target genes does ΔNp63α regulate and which of these genes are coordinately regulated by p53? Our *in vitro* DNA binding assays and ChIP analyses support the hypothesis that p53 and p63 can coordinately regulate target genes such as p21 and 14-3-3σ. If ΔNp63α acts as a transcriptional repressor as our Gal4-fusion experiments show, then protein levels, promoter binding affinity, and co-associated proteins are some of the factors that may be involved in this coordinate regulation. Similar to our results, Weinberg *et al.* reported that p53 transcript and protein decreased during differentiation but p21 promoter activity increased (48). Does this increase reflect an actual elevation in p53 activity or a loss of ΔNp63α repression? Another study showed that p63 decreased in mouse keratinocyte cultures or mouse epidermis exposed to UV-B (29). However, ectopic expression of ΔNp63α in the mouse epidermis resulted in decreased UV-B-induced apoptosis (29). Does this finding represent a ΔNp63α dominant negative effect at p53 target genes involved in the induction of

apoptosis? These observations also raise the possibility that p53 and ΔNp63α compete for consensus DNA binding sites. Based on our *in vitro* binding assays, p53 has a relatively higher binding affinity than ΔNp63α for known p53 binding sites, with the exception of 14-3-3σ site 1. This finding supports the following model. When rapidly proliferating basal epithelial cells are exposed to cell stress, increased p53 protein combined with the higher binding affinity of p53 for select promoter sites causes displacement of the constitutively expressed ΔNp63α. These events lead to subsequent transactivation of genes whose products are involved in growth arrest and apoptosis. This model is also consistent with a role of ΔNp63α overexpression as an oncogenic signal through competition with p53 for select binding sites. In support of this theory, studies by Hibi *et al.* (24) and Choi *et al.* (9) showed p63 gene amplification and protein overexpression in squamous cell carcinomas of the head and neck. Additionally, ectopic expression of the $p40^{AIS}$ splice variant in Rat 1a cells resulted in increased soft agar growth and increased tumor size in mice (24). However, it is likely that p63 also regulates gene expression independently of p53, since mutation of p53 and amplification of p63 both occur during genesis of squamous cell carcinomas (24) (Sniezek and Pietenpol, unpublished results).

Taken together, these data suggest a model in which p63 can bind select promoter elements and repress expression of growth inhibitory genes.

The ability of p63 to function as a transcriptional repressor would allow for continued proliferation of keratinocytes in the basal layer where p63 is localized in stratified epithelium. Further experimentation is required to further link p63 biochemistry to biology, and determine the interplay of p63 and p53 signaling pathways. New technologies including *in vivo* DNA binding assays and mass spectrometry will aid in the identification of key post-translational modifications, associated proteins, and novel target genes that are regulated by p63.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Differentiation-induced modulation of ΔNp63α protein levels in primary HEKs. HEKs were induced to differentiate as described in Materials and Methods. A) Western analysis of HEK lysates from rapidly growing and differentiating cells for expression of terminal differentiation markers loricrin and involucrin. B) Micrograph (32X) of rapidly growing HEKs and HEKs day 4 after induction of differentiation. C) Western analysis for p63 expression in rapidly growing and differentiating HEKs. ΔNp63 splice variants were ectopically expressed in RKO cells and lysates analyzed to serve as molecular weight markers for comparison to HEK p63. The asterisk to the left of the blot aligns with a cross-reactive band that may represent ΔNp63β expression. D) Northern analysis of p63 transcript expression in HEKs differentiated as panel A. Results show one representative result of three independent experiments with separate primary cultures of HEKs.

Figure 2. ΔNp63α is a phosphoprotein. A) Western analysis of differentiating HEKs showing several forms of ΔNp63α on one-dimensional SDS-PAGE (arrows denote three differentially migrating ΔNp63α forms). B) Western analysis of potato acid phosphatase (PAP) treated HEK lysates from day 4 after differentiation. Each lysate was incubated with PAP as

described in Materials and Methods. Results show one representative result of four independent experiments.

Figure 3. Differentiation-induced changes in select protein and mRNA levels in primary human epidermal keratinocytes. HEKs were induced to differentiate as described in Materials and Methods. A) Western analysis of p63, p53, 14-3-3σ, and p21 in rapidly growing and differentiating HEKs (note that p63 migrates as a single band because gels were run for shorter times than in Fig. 1 and 2 to include allow for analysis of the molecular-weight range of proteins shown in panel A). The numbers below the Western panels represent fold change relative to rapidly growing HEKs. B) Northern analysis of transcripts for proteins shown in A. Results show one representative result of three independent experiments with separate cultures of primary HEKs.

Figure 4. ΔNp63α Represses Transcription. A) Schematic representing vectors used in panels: B, C, and D. Abbreviations are UAS, upstream activating sequence; DBD, DNA binding domain; TK, thymidine kinase; and SAM, sterile alpha motif. SAM mutants are as follows: L459F, G475V, T478P, and Q481L. B) Analysis of Gal4 fusion proteins. HCT116 cells were transfected with a p21-luciferase reporter construct and either

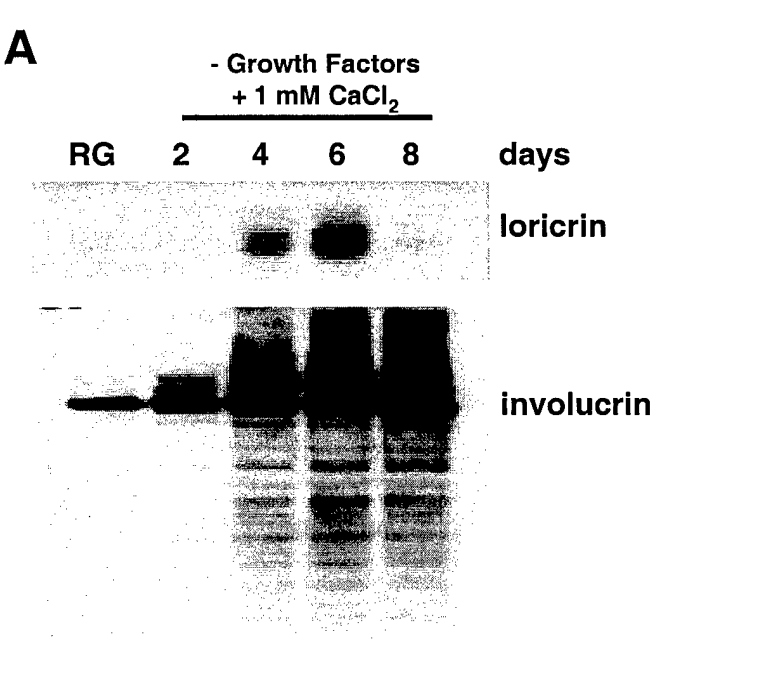
p53, ΔNp63α, or increasing amounts of Gal4-ΔNp63α. C) 293 cells were transfected with the luciferase reporter vector in panel 4A and the indicated Gal4 fusion vectors. Gal4 alone served as the negative control and Gal4-ETO2 served as the positive control. D) 293 cells were transfected with the murine Gal4-ΔNp63α and the indicated SAM domain point mutants. All luciferase assays were normalized for transfection efficiency with a renilla reporter vector. Results are representative of one experiment, performed in triplicate, of five independent experiments.

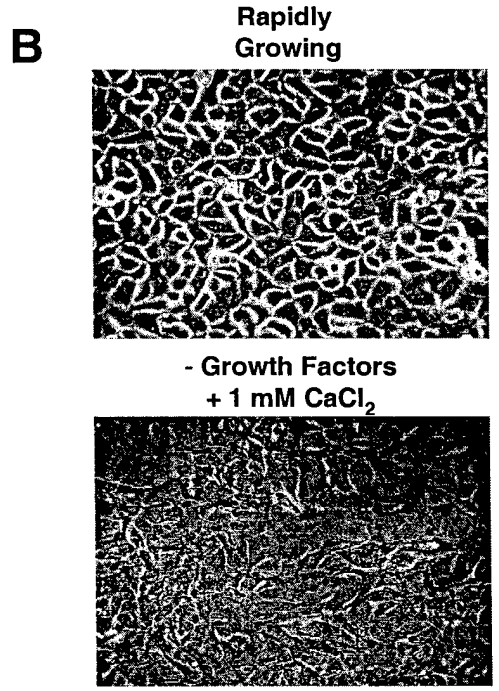
Figure 5. Relative Binding Affinities of p53 and ΔNp63α for p53 Consensus Sites in the p21 and 14-3-3σ Promoters. A) The p53 consensus binding sequence and p53 binding sites in the p21 and 14-3-3σ promoters. Abbreviations are R, purine; Y, pyrimidine; and W, A or T. B) H1299 cells were transfected with myc-tagged p53 or ΔNp63α and protein lysates quantified using the Fluor-S Max MultiImager. Based on Fluor-S Max quantification, equal amounts of myc-tagged p53 and myc-tagged ΔNp63α were immunoprecipitated with a myc-epitope antibody. Immunoprecipitated p53 and ΔNp63α were assayed for their ability to bind radiolabeled oligonucleotides representing p53 binding sites in the p21 and 14-3-3σ promoters as described in Materials and Methods. Bound

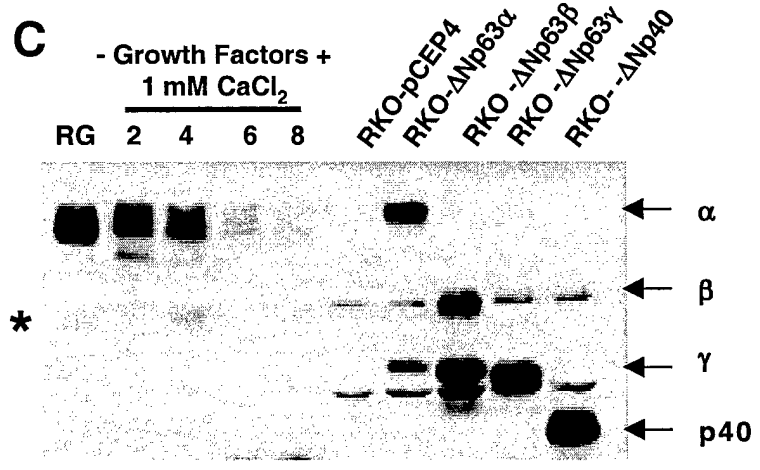
oligonucleotides were separated on acrylamide gels, exposed for autoradiography, and quantified using an Instant Imager (C). Each autoradiograph shows one representative result of at least three independent experiments that are quantified and displayed with standard deviation in panel C.

Figure 6. ChIP Analyses for p53 and p63 binding at the 14-3-3σ and p21 promoters during keratinocyte differentiation. Abbreviations are (RG), Rapidly Growing; (8), Day 8 after induction of differentiation; C, control. HEKs were induced to differentiate as described in Materials and Methods. At the time of harvest, HEKs were treated with formaldehyde (X-Link) and processed as described in Materials and Methods. The DNA immunoprecipitated with p53- or p63-specific antibodies was PCR amplified using primers flanking the p53 binding sites in the p21 (A and C), and 14-3-3σ (E and G) promoters. DNA fragments generated by PCR were resolved by PAGE, the gels were stained with ethidium bromide, and the PCR products were quantified by densitometry. Data are expressed as fold of the rapidly growing (X-Link) DNA level. For p21 and 14-3-3σ "+" indicates PCR products that were generated using DNA template derived from total genomic DNA harvested from rapidly growing HEKs and "–" indicates no DNA input for the PCR reaction. Each ethidium bromide-stained gel shows one

representative result of at least three independent experiments that are quantified and displayed with standard deviation in panels B, D, F, and H.


A
- Growth Factors
+ 1 mM CaCl2
RG 2 4 6 8 days
loricrin
involucrin


B
Rapidly
Growing
- Growth Factors
+ 1 mM CaCl2


C
- Growth Factors +
1 mM CaCl2
RG 2 4 6 8
RKO-pCEP4
RKO-ΔNp63α
RKO -ΔNp63β
RKO -ΔNp63γ
RKO- -ΔNp40
α
β
γ
p40
*

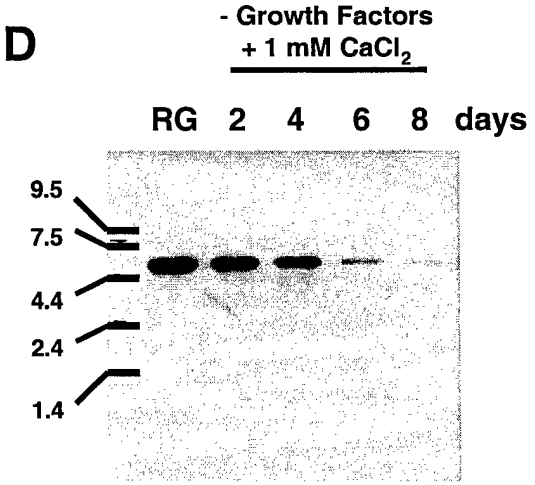

D
- Growth Factors
+ 1 mM CaCl2
RG 2 4 6 8 days
9.5
7.5
4.4
2.4
1.4

Figure 1

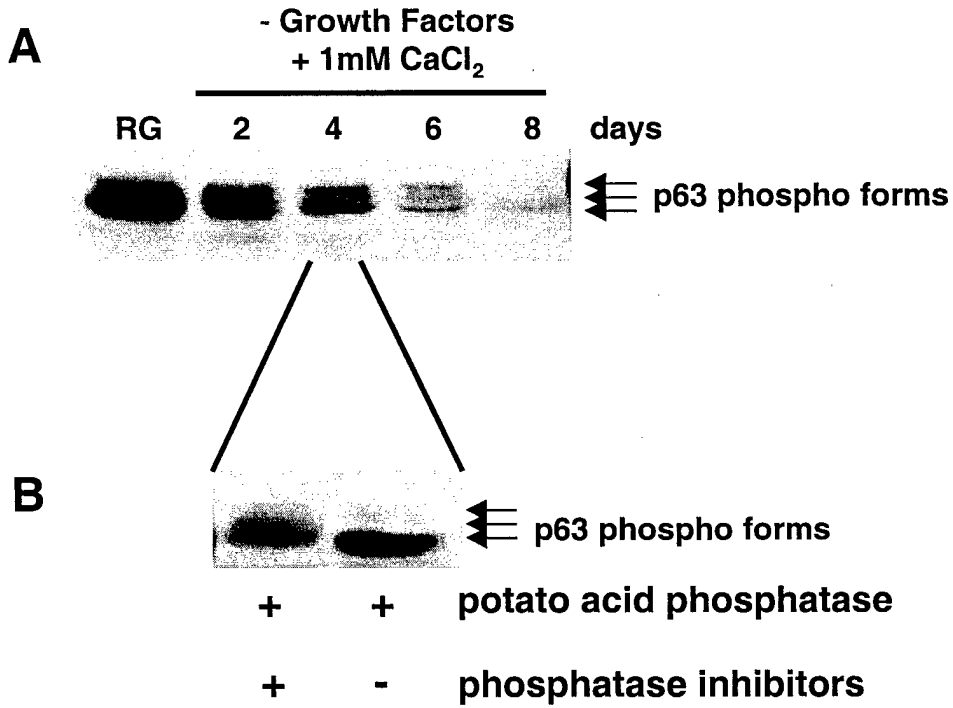

A
- Growth Factors
+ 1mM CaCl2
RG
2
4
6
8
days
p63 phospho forms
B
p63 phospho forms
+
+
potato acid phosphatase
+
-
phosphatase inhibitors

Figure 2

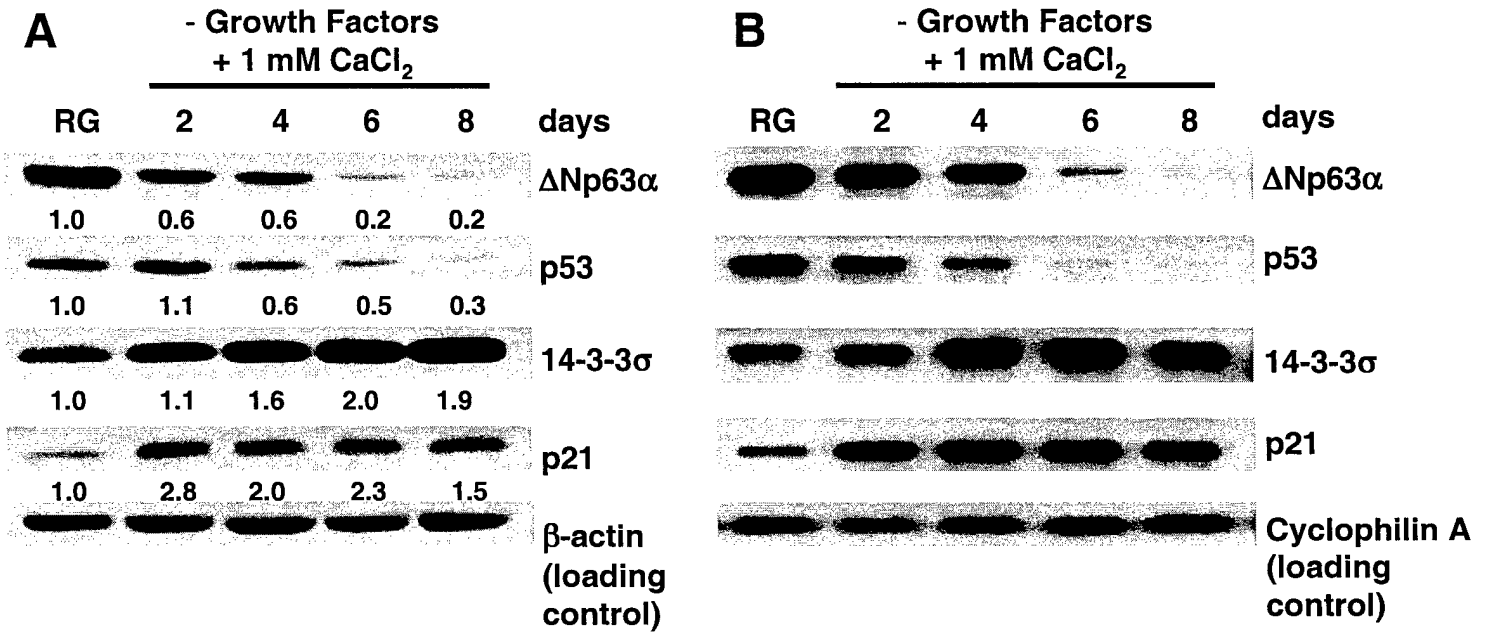
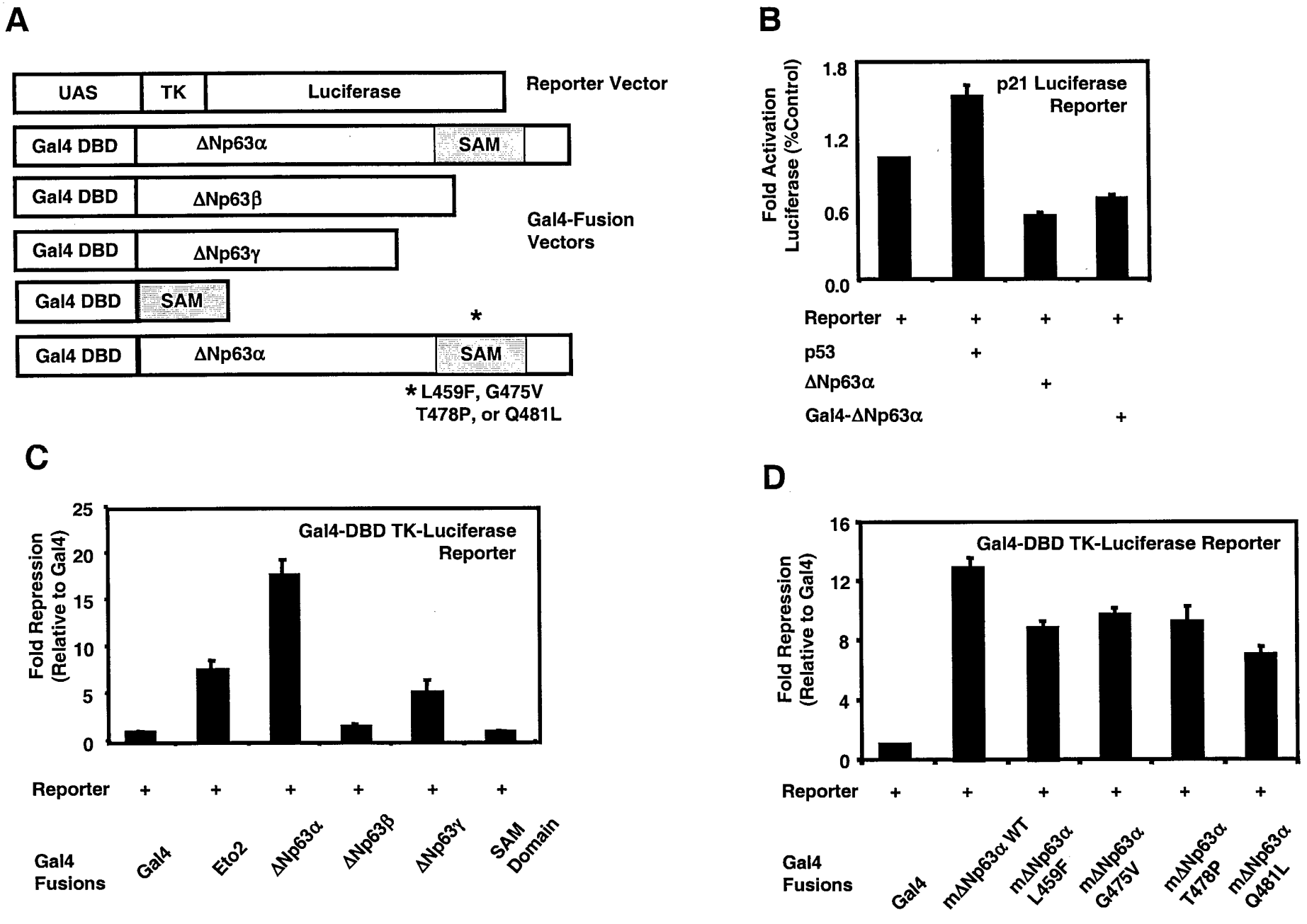

A
- Growth Factors
+ 1 mM CaCl2
RG
2
4
6
8
days
ΔNp63α
1.0
0.6
0.6
0.2
0.2
p53
1.0
1.1
0.6
0.5
0.3
14-3-3σ
1.0
1.1
1.6
2.0
1.9
p21
1.0
2.8
2.0
2.3
1.5
β-actin
(loading
control)
B
- Growth Factors
+ 1 mM CaCl2
RG
2
4
6
8
days
ΔNp63α
p53
14-3-3σ
p21
Cyclophilin A
(loading
control)

Figure 3

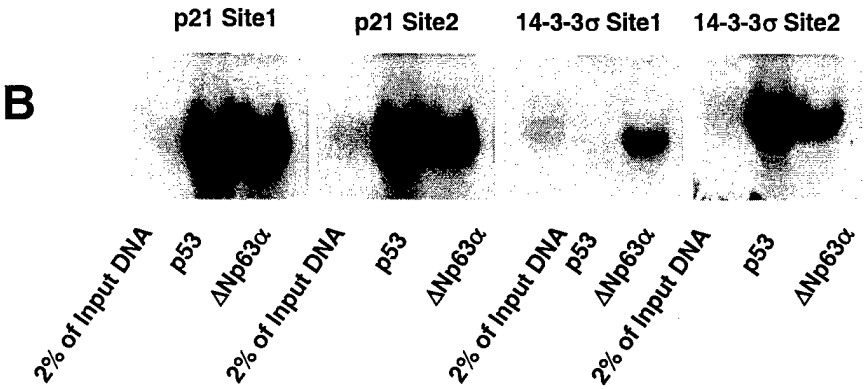
Figure 4


A
UAS
TK
Luciferase
Reporter Vector
Gal4 DBD
ΔNp63α
SAM
Gal4 DBD
ΔNp63β
Gal4 DBD
ΔNp63γ
Gal4-Fusion Vectors
Gal4 DBD
SAM
*
Gal4 DBD
ΔNp63α
SAM
* L459F, G475V T478P, or Q481L
B
Fold Activation Luciferase (%Control)
1.8
1.2
0.6
0.0
p21 Luciferase Reporter
Reporter + + + +
p53 +
ΔNp63α +
Gal4-ΔNp63α +
C
Fold Repression (Relative to Gal4)
25
20
15
10
5
0
Gal4-DBD TK-Luciferase Reporter
Reporter + + + + + +
Gal4 Fusions
Gal4
Eto2
ΔNp63α
ΔNp63β
ΔNp63γ
SAM Domain
D
Fold Repression (Relative to Gal4)
16
12
8
4
0
Gal4-DBD TK-Luciferase Reporter
Reporter + + + + + +
Gal4 Fusions
Gal4
mΔNp63α WT
mΔNp63α L459F
mΔNp63α G475V
mΔNp63α T478P
mΔNp63α Q481L

A

p53 consensus	RRRCWWGYYYRRRCWWGYYY
p21 site 1	GAACATGTCCCAACATGTTG
p21 site 2	AGACTGGGCATGTCTGGGCA
14-3-3σ site 1	AGGCATGTGCCACCATGCCC
14-3-3σ site 2	GTAGCATTAGCCCAGACATGTCC

B



C

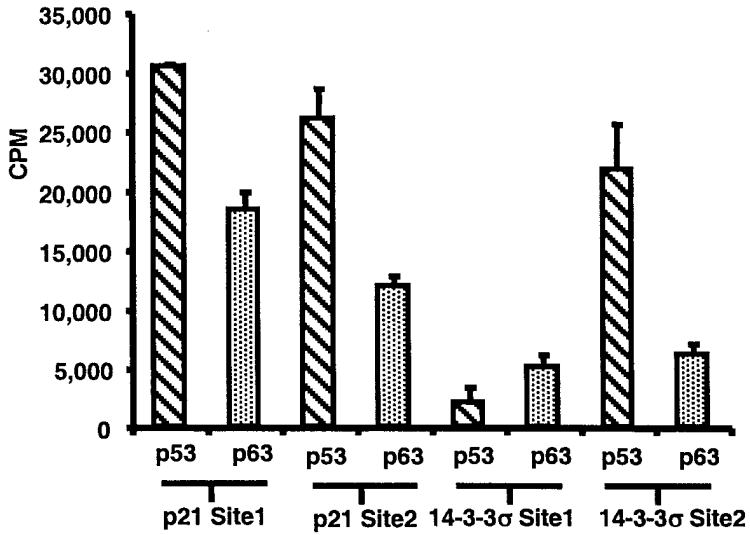


Figure 5

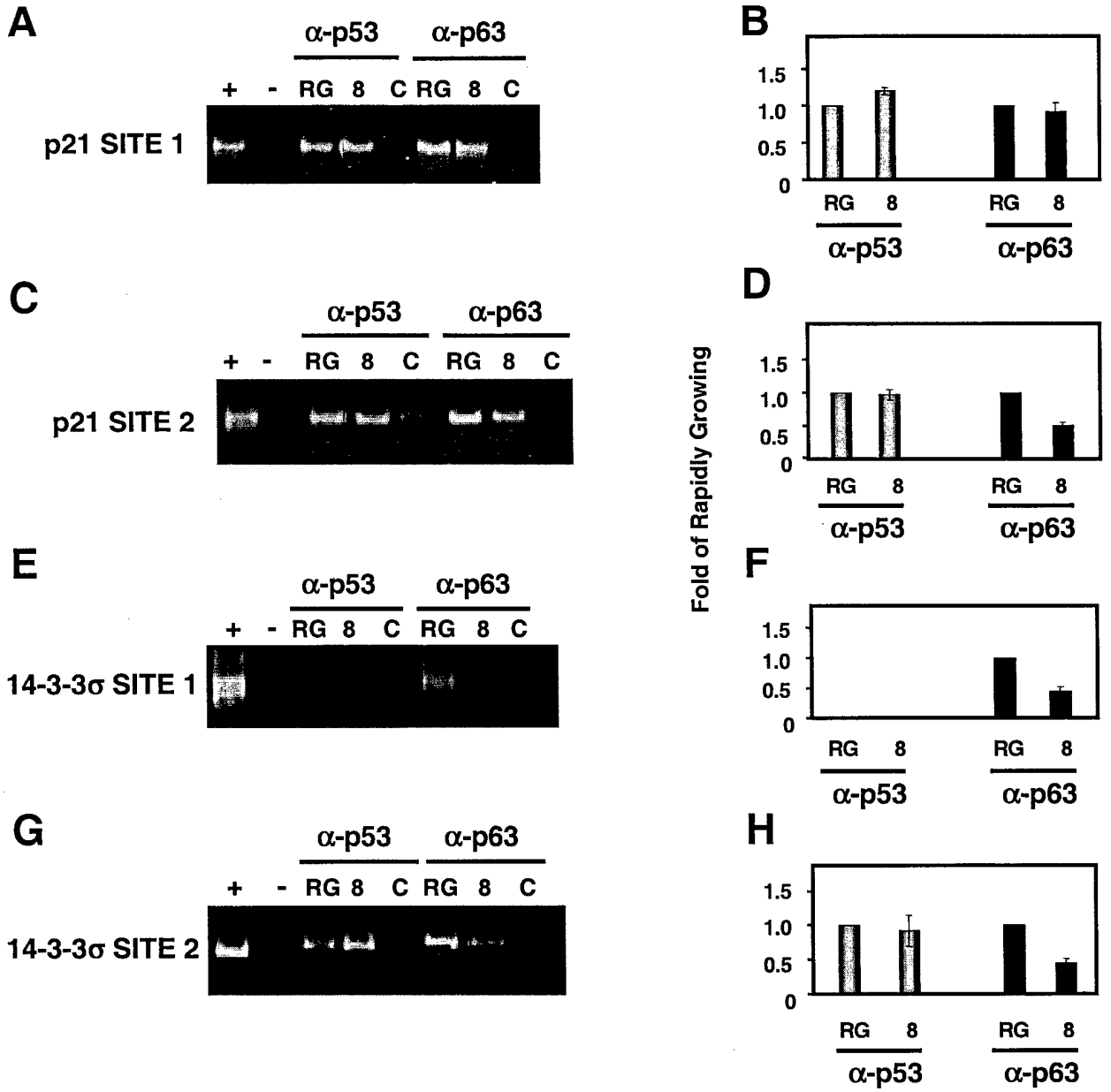

A
α-p53
α-p63
+ - RG 8 C RG 8 C
p21 SITE 1
B
1.5
1.0
0.5
0
RG 8 RG 8
α-p53 α-p63
C
α-p53
α-p63
+ - RG 8 C RG 8 C
p21 SITE 2
D
Fold of Rapidly Growing
E
α-p53
α-p63
+ - RG 8 C RG 8 C
14-3-3σ SITE 1
F
G
α-p53
α-p63
+ - RG 8 C RG 8 C
14-3-3σ SITE 2
H

Figure 6